(12) PATENT
(19) AUSTRALIAN PATENT OFFICE

(11) Application No. AU 199478159 B2
(10) Patent No. 709336

(54) Title
Anti-cancer gene therapy by modulation of immune or inflammatory response

(51)[6] International Patent Classification(s)
C12N 015/86 C07K 014/55
A61K 048/00 C12N 015/24
C07K 014/54 C12N 015/26

(21) Application No: 199478159 (22) Application Date: 1994.09.28

(87) WIPO No: WO95/09241

(30) Priority Data

(31) Number	(32) Date	(33) Country
93/11601	1993.09.29	FR

(43) Publication Date : 1995.04.18
(43) Publication Journal Date : 1995.06.08
(44) Accepted Journal Date : 1999.08.26

(71) Applicant(s)
Transgene S.A.

(72) Inventor(s)
Bruce Acres

(74) Agent/Attorney
CARTER SMITH and BEADLE,Qantas House,2 Railway Parade,CAMBERWELL VIC 3124

(56) Related Art
TARTAGLIA J ET AL. (1990) CRITICAL REVIEWS IN IMMUNOLOGY 10(1):13-30

OPI DATE 18/04/95 APPLN. ID 78159/94
AOJP DATE 08/06/95 PCT NUMBER PCT/FR94/01133
AU9478159

DEI.

(51) Classification internationale des brevets 6 : C12N 15/86, A61K 48/00, C12N 15/24, 15/26, C07K 14/55, 14/54 — A2

(11) Numéro de publication internationale: WO 95/09241

(43) Date de publication internationale: 6 avril 1995 (06.04.95)

(21) Numéro de la demande internationale: PCT/FR94/01133

(22) Date de dépôt international: 28 septembre 1994 (28.09.94)

(30) Données relatives à la priorité:
93/11601 29 septembre 1993 (29.09.93) FR

(71) Déposant: TRANSGENE S.A. [FR/FR]; 11, rue de Molsheim, F-67000 Strasbourg (FR).

(72) Inventeur: ACRES, Bruce; 20, rue L.-Apffel, F-67000 Strasbourg (FR).

(74) Mandataire: WARCOIN, Jacques; Cabinet Regimbeau, 26, avenue Kléber, F-75116 Paris (FR).

(81) Etats désignés: AU, JP, brevet européen (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Publiée
Sans rapport de recherche internationale, sera republiée dès réception de ce rapport.

(54) Title: ANTI-CANCER GENE THERAPY BY MODULATION OF IMMUNE OR INFLAMMATORY RESPONSE

(54) Titre: THERAPIE GENIQUE ANTICANCEREUSE PAR MODULATION DE LA REPONSE IMMUNITAIRE ET/OU INFLAMMATOIRE

(57) Abstract

A viral vector in whose genome is inserted a DNA fragment comprising one or more genes coding for all or part of an immune and/or inflammatory response modulating gene, is used in the preparation of a drug for the treatment of cancers in mammals. The invention concerns specifically the use of a viral vector derived from a poxvirus in whose genome is inserted a gene coding for a cytokin, in particular, interleukine-2, 4, 5, 6 or 7, gamma interferon, the colony stimulating factor or type β tumour necrosis factor.

(57) Abrégé

La présente invention a trait à l'usage d'un vecteur viral dans le génome duquel est inséré un fragment d'ADN comportant un ou plusieurs gènes codant pour tout ou partie d'un agent modulateur de la réponse immunitaire et/ou inflammatoire, pour la préparation d'un médicament pour le traitement des cancers chez les mammifères. Elle concerne plus particulièrement l'usage d'un vecteur viral dérivé d'un poxvirus dans le génome duquel est inséré un gène codant pour une cytokine, notamment une interleukine-2, 4, 5, 6 ou 7, l'interféron gamma, le facteur stimulant les colonies ou le facteur nécrosant les tumeurs de type β.

ANTICANCER GENE THERAPY BY MODULATION OF THE IMMUNE AND/OR INFLAMMATORY RESPONSE

The present invention relates to a method for the treatment of cancer by gene therapy. It relates more especially to the use of a viral vector in order to deliver to tumour cells a gene coding for an agent that modulates the immune and/or inflammatory response.

It is generally accepted that cancer is a disease which results from a loss of the control of cell multiplication. Its causes may be numerous and due, in particular, to a defect of the functioning of cellular genes (activation of potentially oncogenic genes, for example by somatic mutation of normal genes; deregulation of the expression of cellular genes; inhibition of the expression of tumour-suppressing genes) or to the undesirable expression of viral genes.

During the last 20 years, it has been demonstrated that most tumour cells present at their surface tumour-specific antigens (non-self antigens) which do not find their equivalent in normal cells. These tumour-specific antigens are, for example, (i) cellular antigens whose expression takes place during the foetoembryonic period and regresses at birth to the point where it disappears, (ii) antigens which are normally expressed at a very low level and which, when expressed at high level, become characteristic of a tumour, or (iii) cellular antigens whose structure or conformation is modified.

In principle, the aberrant expression of these tumour-specific antigens is capable of triggering an immune response of the same type as that induced by any non-self antigen. This response brings into play all of the cells of the immune system, including the neutrophils, lymphocytes, monocytes and macrophages.

Generally speaking, there are two major types of immune response: the humoral type response, which corresponds to the production of antibodies by B lymphocytes, and the cell-mediated immune response with a cytotoxic effect, which involves effector cells, essentially

cytotoxic T (T_c) lymphocytes, NK (natural killer) cells and phagocytic cells. Regulatory cells modulate both types of immune response: essentially T helper (T_H) lymphocytes and T suppressor (T_S) lymphocytes.

An immune response is an extremely complex phenomenon which requires, in particular, the cooperation of different cell types. This cooperation comes about through the intermediary action of cytokines, which are soluble molecules which participate as cell to cell mediators.

As regards humoral immunity, the B lymphocytes are stimulated by non-self antigens in their native conformation. In response to this stimulation, the B lymphocytes produce specific antibodies directed against these foreign antigens.

The T lymphocytes, on the other hand, can be stimulated only by peptides, degradation products of the non-self antigens, presented at the surface of antigen-presenting cells (APC) in association with antigens of the major histocompatibility complex (MHC).

The activation of T lymphocytes has the effect of triggering their amplification and bringing their functions into play; in particular, the destruction of infected or tumour cells by cytotoxic lymphocytes.

Only one class of antigens, designated superantigens, is not presented in a conventional manner. In effect, superantigens are capable of binding to molecules of the MHC without being degraded beforehand to peptides, and can simultaneously activate a larger amount of T lymphocytes than would be activated by the pathway of classical antigens. These superantigens are hence capable of inducing a strong immune response.

Inflammation is triggered by the body's response to damage, for example a wound or an infection. The inflammatory response comprises a whole series of reactions including, in particular, the release of chemotactic or chemoattractant molecules (also designated by the term chemokines) which have the effect of attracting the cells of the immune system to the actual site of

inflammation.

Activated T lymphocytes produce, inter alia, molecules that inhibit cell migration phenomena, such as MIF (migration inhibition factor). As its name indicates, MIF has the role of inhibiting the migration of macrophages, and consequently of promoting their concentration at the site of inflammation, for which they exert their function of phagocytosis under optimal conditions.

In the case of a declared cancer, the antitumour immune response is deficient, either because the immune system itself is deficient, or because the phenotypic changes in the tumour cells inhibit or do not suffice to trigger the immune response.

In order to treat cancers, it has already been proposed to strengthen the antitumour immune response by administering to patients systemic and repeated doses of cytokines such as interleukin-2 (IL-2) interferon (IFN-γ) or tumour necrosis factor (TNF) type α (Rosenberg, 1992, J. Clin. Oncology, *10*, 180-199). Unfortunately, the side effects are not insignificant, ranging from nausea even to death. Furthermore, such a treatment proves extremely expensive.

Similar thinking lies behind an alternative method which has also been proposed, based on the *ex vivo* transfer of a gene coding for an immunostimulatory molecule into a patient's cells; this being done for purposes of expression. Briefly, (i) tumour cells or tumour infiltrating lymphocytes (TIL) are removed from a patient; (ii) they are transfected *ex vivo* with a vector carrying a gene coding for an immunostimulatory molecule such as IL-2, IL-4, IL-6 and TNFα; and (iii) they are reimplanted into the patient from whom they originated.

As before, clinical trials have, to date, given only modest or only disappointing results. Many investigators report a low level of expression (Anderson, 1993, Science, *259*, 1391-1392). Furthermore, such a protocol is difficult to apply on a large scale. In effect, it necessitates the bulk culture of cells for each patient to be treated, with the drawbacks this involves from a

cost, time and risk standpoint. In addition, the absence of expression of new tumour antigen variants during the *in vitro* culture phase cannot be guaranteed.

Very recently, Plautz et al. (1993, Proc. Natl. Acad. Sci. USA, 90, 4645-4649) have reported the direct *in vivo* transfection of mouse tumour cells with a recombinant retrovirus. The latter was modified in order to permit the expression of a complementary DNA (cDNA) coding for a mouse MHC surface antigen. The antigen selected is allogeneic, that is to say it displays a genetic variation with respect to the host mouse, with the object of stimulating the immune response to tumour cells expressing this antigen. While this method eliminates the need to establish a cell line for each patient, it is, however, applicable only to tumours which are accessible by surgery.

It has now been found that a vaccinia virus administered to a mouse developing a tumour preferentially infects the cancerous tissues. When the vaccinia virus carries a gene coding for an immunostimulatory molecule, an inhibition of tumour growth, and in some cases a complete regression, is observed.

Thus, the subject of the present invention is the use of a viral vector into the genome of which is inserted a DNA fragment containing one or more genes coding for at least one agent participating in the destruction of cancer cells, e.g. an agent which is toxic to cancer cells or an agent that modulates the immune and/or inflammatory response; for the preparation of a medicinal product for the treatment of cancer in mammals.

Accordingly, in one aspect the invention provides use of a viral vector in the genome of which is inserted a DNA fragment containing one or more genes coding for all or part

of an agent that modulates the immune and/or inflammatory response, wherein said agent is:

of cell origin;

selected from cell surface costimulatory molecules, chemokines, monoclonal antibodies directed against lymphocytes surface markers, superantigens characteristic of an infectious organism (bacterium, virus or parasite) and polypeptides having an adjuvant function, for the preparation of a medicinal product for parenteral administration, and in particular intravenous or intramuscular administration, for the treatment of a declared cancer in mammals.

"Viral vector" is understood to mean a virus whose genome has been modified so as to permit the transfer of a gene of interest into a eukaryotic cell and its expression therein. A viral vector which can be used in the context of the present invention may be derived, in particular, from a poxvirus, a herpesvirus, a retrovirus or an adenovirus. Advantageously, the vector in question will be non-integrative and non-replicative, and one for which the host cell or origin is non-human,

such as, for example, the canarypox virus. Such vectors as well as the techniques for preparing them are known to a person skilled in the art.

As regards a viral vector derived from an adenovirus, this will preferably consist of the complete genome of the adenovirus at least lacking E1A gene located at the 5' end and coding for a transactivating protein essential to the replication of the adenovirus. It will hence be propagated in a complementation cell line supplying in *trans* the expression product of the E1A gene. It is self-evident that other regions of the adenoviral genome may be modified or deleted, especially the non-essential E3 region and, alternatively, the other regions essential to viral replication, inasmuch as the lacking functions are complemented in *trans*. Such a vector will nevertheless comprise the sequences essential to encapsidation, namely the 5' and 3' ITRs (inverted terminal repeats) and the encapsidation region. The various adenoviral vectors as well as the techniques for preparing them are conventional and are presented in Graham and Prevect (Methods in Molecular Biology, vol 7, p 109-128; Ed: E.J. Murey, The human Press Inc.).

According to an especially preferred embodiment, a viral vector which is useful for the purposes of the present invention is derived from a poxvirus, in particular from a vaccinia virus or from an avian poxvirus such as the canarypox virus; the latter being preferred.

The general conditions for obtaining a vaccinia virus capable of expressing a heterologous gene are described in European Patent EP 83,286 and Application EP 206,920. According to an advantageous embodiment, the said DNA fragment will be inserted into the TK gene of the vaccinia virus so as to inactivate the viral gene and to facilitate the selection of the recombinant vaccinia viruses.

In accordance with the objectives towards which the present invention is directed, a viral vector can, in addition, comprise a block for the expression of a selectable marker gene so as to facilitate the steps of

isolation and purification of the recombinant virus. There may be mentioned, in particular, the *Neo* gene conferring resistance to the antibiotic G418, or the herpes simplex type 1 virus (HSV-1) TK gene which confers sensitivity to certain nucleoside analogues such as ganciclovir or acyclovir.

Such a viral vector may also include a gene other than the ones defined above, which can act cooperatively with the desired modulatory effect on the immune and/or inflammatory reaction. The gene in question will advantageously be one coding for all or part of a tumour-specific antigen. There may be mentioned, more especially, E6 and E7 proteins of the HPV virus, in particular type 16 or 18, which are involved in cancers of the uterus, the MUC1 protein, and more especially the repeat region of the latter, involved in breast cancers, and lastly the GA.733.2 antigen involved in colorectal cancers. The sequences coding for the antigens are described in the prior art. It is within the capacity of a person skilled in the art to isolate them, for example by the PCR (polymerase chain reaction) technique employing complementary primers, to insert them into the selected viral vector upstream or downstream of the modulatory gene and to place them under the control of the elements needed for their expression.

For the purposes of the present invention, a DNA fragment containing one or more genes coding for all or part of an agent that modulates the immune and/or inflammatory response, hereinafter designated modulatory agent, is introduced into a viral vector which is a vehicle for the transfer and expression of the said gene(s). The methods for inserting a DNA fragment into a viral vector are known to a person skilled in the art.

Moreover, the gene coding for a modulatory agent can be of the genomic type (containing all or part of the set of introns of the natural gene), of the complementary DNA (cDNA) type lacking introns or of the minigene type, that is to say mixed type containing at least one intron. It can code for a native modulatory agent such as is

found in a mammal, for a portion of such an agent, for a chimeric molecule originating from the fusion of sequences of diverse origins or a mutant displaying improved or modified biological properties, provided, however, that these molecules exert an immunomodulatory function or a modulatory function on the inflammatory response. Such a mutant may be obtained by mutation, deletion, substitution and/or addition of one or more nucleotide(s) of the gene coding for the said modulatory agent. The gene in question can code for (i) a soluble molecule, either intracellular or secreted into the external medium, or (ii) a molecule anchored in the membrane and hence present at the surface of the cells which express it.

The genes coding for a modulatory agent may be obtained by cloning, by PCR or by chemical synthesis according to the conventional techniques in common use.

Naturally, the DNA fragment can comprise the appropriate elements for regulation of transcription, as well as translation initiation and termination signals permitting the expression of the gene or genes coding for a modulatory agent. Among these elements, the promoter region assumes special importance.

Generally speaking, use will be made of a promoter region which is functional in the cells of the mammal which it is desired to treat, preferably in human cells. It can be the promoter region naturally governing the expression of the said gene, or a promoter region of different origin, for example originating from eukaryotic or viral genes. Moreover, the promoter region may be modified so as to contain regulatory sequences, for example a transcription activating element (enhancer) or sequences responding to certain cellular signals.

The promoter region selected may be constitutive or regulable, and, in the latter case, regulable in response to certain tissue-specific or event-specific cellular signals. It will be advantageous to use a tissue-specific promoter region when the tumour to be treated originates from a particular cell type. Alterna-

tively, the use of a promoter responding to specifically tumoral signals (for example regulable by the presence of growth factors generally released by tumour cells) may prove advantageous, since the expression will be limited to tumour cells.

Such promoters are generally known to a person skilled in the art. There may be mentioned, in particular, the SV40 (simian virus 40), HMG (hydroxymethylglutarylcoenzyme A) and TK (thymidine kinase) promoters, the RSV (Rous sarcoma virus) and Mo-MLV (Moloney murine leukaemia virus) LTRs (long terminal repeats), the adenovirus MLP (major late promoter), the vaccinia virus 7.5K and H5R promoters, the liver-specific promoters of the α_1-antitrypsin, albumin, coagulation factor IX and transferrin genes and the promoters of the immunoglobulin genes which permit the expression of the genes they govern in lymphocytes. These examples are not limiting.

In the context of the present invention, a DNA fragment can contain one or more gene(s) coding for a modulatory agent, which may be placed under the control of the elements permitting their expression, independently or jointly. In other words, the DNA fragment may contain one or more cassettes for the expression of one or more genes coding for a modulatory agent.

Moreover, the DNA fragment may also include a signal sequence permitting the secretion of the modulatory agent in question out of the cell. It can be a natural signal sequence of the gene coding for the said modulatory agent, or alternatively a heterologous signal sequence which is functional in eukaryotic cells, for example the signal sequence of the gene coding for transferrin or α_1-antitrypsin.

Agent that modulates the immune and/or inflammatory response is understood to means any molecule capable, in particular, of:

- stimulating a humoral immune response, by activating the B lymphocytes so as to amplify the production of antibodies directed against tumour-specific antigens;

- stimulating a cell-mediated immune response, by activating the T lymphocytes so as to trigger a significant cytotoxic or delayed type hypersensitivity (DTH) response to tumour cells;

- inducing or stimulating an inflammatory response so as to channel the cells of the immune system to the tumour site; or

- inhibiting cell migration phenomena so as to preserve the cells of the immune system at the actual tumour site or in proximity to a tumour site.

A molecule displaying or more of the functions defined above may be selected, in particular, from (I) cytokines, (II) cell surface costimulatory molecules, (III) chemokines, (IV) monoclonal antibodies directed against lymphocyte surface markers, (V) superantigens characteristic of an infectious organism (bacterium, virus or parasite) and (VI) polypeptides having an adjuvant function.

(I)

Advantageous cytokines for the purposes of the present invention are those produced by the cells of the immune system, in particular lymphocytes and macrophages or alternatively their progenitor stem cells, and which participate in the activation of the cells of the immune system, the transport of signals between the cells of the immune system and the cellular differentiation of the stem cells into mature cells of the blood stream.

By way of cytokines which are useful for the purposes of the present invention, there may be mentioned in particular:

(1) Interleukins (IL). At the present time, 16 interleukins have been identified. It is especially difficult to assign them a specific function since they exert pleiotropic effects. In the context of the present invention,

all the interleukins are of interest, but the following may be mentioned more especially:

- IL-1, which is produced by macrophages following a stimulation by bacterial components. Its role is exerted at the level of lymphocyte activation. IL-1 is also a pro-inflammatory molecule and, as such, induces chemokine production.

- IL-2, which is responsible for the proliferation of activated T lymphocytes and which, in combination with IFN-γ, stimulates antibody production by B lymphocytes. Furthermore, some studies tend to show that it has a chemotactic role for lymphocytes when it is produced in a tumour.

- IL-4, which is produced by activated T lymphocytes and stimulates the growth of B lymphocytes and, in some cases, of T lymphocytes.

- IL-5, which promotes the multiplication and differentiation of eosinophil leucocytes as well as, to a lesser extent, antibody production.

- IL-6, which is produced by macrophages and T lymphocytes. It has a pleiotropic effect and participates, inter alia, as a stimulant of the cytotoxic activity of T lymphocytes and of antibody production. It is also a pro-inflammatory molecule.

- IL-7, which is produced by cells of the stroma of the bone marrow and participates in the proliferation of pre-B and -T lymphocytes.

- IL-12, which is produced by activated macrophages and induces IFN-γ production.

(2) Interferons (IFN), which possess antiviral and immunomodulatory properties. They can activate the phagocytic cells and enhance the expression of the MHC

class I and II surface antigens, and can also stimulate the cytotoxicity of NK cells against tumour cells. There are three main classes of IFNs, each being of interest in the context of the present invention. These different classes, α, β and γ, respectively, each comprise many subtypes.

(3) Colony stimulating factors CSF, which participate in the maturation of the haematopoietic stem cells and their differentiation into mature cells of the blood stream. GM-CSF, G-CSF and M-CSF (for granulocyte-macrophage, granulocyte and macrophage, respectively) are distinguished according to the stage of maturation and to the cell type on which these factors exert their influence.

(4) Tumour necrosis factors (TNF). TNFα produced by macrophages and TNFβ produced by T lymphocytes are distinguished. Both are responsible for the antitumour cytotoxic activity of macrophages and lymphocytes and for the local tissue changes observed in the inflammatory reaction.

(5) MIF factors, which inhibit macrophage migration.

(6) Complement fragment C5a, which is a chemotactic factor for macrophages.

For the purposes of the present invention, IL-2, IL-4, IL-5, IL-6, IL-7, IFN-γ, GM-CSF and TNFβ are most especially preferred.

(II)

Costimulatory molecules are molecules present at the cell surface which participate indirectly in the immune and/or inflammatory reaction; for example, molecules participating in the process of presentation of the antigen in a form which is recognizable by the cells of the immune system.

By way of costimulating molecules which are

useful for the purposes of the present invention, the following may be mentioned in particular:

(1) Antigens of the major histocompatibility complex (MHC). In man, most nucleated cells express variable amount of class I antigens at their surface, whereas the class II antigens have a distribution restricted to B lymphocytes, phagocytic cells and activated T lymphocytes. These cell surface markers are involved in the phenomena of immune recognition, in particular, as has been described above, in the interaction between T lymphocytes and APC cells and between T_c lymphocytes and target cells presenting non-self antigens at their surface. Hence this suggests that the intensity of the immune response may be improved by increasing the expression of the MHC antigens at the tumour site.

(2) Ligands for the human markers CD27, CD28, CD30 and CD40. In particular, the ligand for the marker CD40 is located at the surface of activated T lymphocytes, and is involved in the stimulation and proliferation of B lymphocytes. On the other hand, *in vitro* studies have shown that the ligands for the markers CD27 and CD30 induce the proliferation of T lymphocytes. As regards the ligand for the marker CD28, also designated protein B7 by some authors, this is synthesized by the APC and participates in the recognition of target cells presenting non-self antigens at their surface by NK cells, thereby permitting their destruction.

(3) The lymphocyte function antigen type 1 LFA-1 (for leucocyte function antigen). This is a surface receptor common to all leucocytes and also present on macrophages, which plays an important part in non-specific cell adhesion phenomena. The latter are involved in the process of chemotaxis, by participating in the migration of cells of the immune system towards an inflammatory site.

(4) The intercellular adhesion molecule type 1 (ICAM-1) present at the surface of many cell types. ICAM-1 is one of the ligands for the LFA-1 molecule.

(III)

As stated above, chemokines are molecules synthesized in proximity to or at an inflammatory site, and which channel the cells of the immune system to this site. Generally speaking, chemokines act through interactions between, on the one hand adhesion molecules present at the surface of leucocytes, and on the other hand adhesion molecules present at the surface of endothelial cells (for example LFA-1 and ICAM-1). The attachment of leucocytes to endothelial cells of the vascular wall is followed by their migration towards the inflammatory site.

By way of chemokines which are useful for the purposes of the present invention, the following may be mentioned in particular:

(1) PF-4 (platelet factor-4; Denel et al., 1977, Proc. Natl. Acad. Sci. USA, *74*, 2256);

(2) RANTES (a chemotactic factor for various types of leucocyte (Schall et al., 1988, J. Immunol., *141*, 1018); and

(3) ACT-2 (Ziptel et al., 1989, J. Immunol., *142*, 1587). This is a protein which promotes the adhesion of T lymphocytes participating in the recognition of the antigen associated with MHC class I molecules.

(IV)

A monoclonal antibody which is useful for the purposes of the present invention is, for example, directed against the cell surface marker CD2, CD3, CD28 or CD40. In effect, some studies have shown the role of

such an antibody in the stimulation of T lymphocyte proliferation.

(V)

A superantigen which is useful for the purposes of the present invention is, for example, the rabies virus nucleoprotein or a bacterial enterotoxin produced, in particular, by a bacterium of the genus *Staphylococcus*.

(VI)

An adjuvant of polypeptide nature which is useful for the purposes of the present invention is, for example, an expression product of a 20S "layer" bacterial gene.

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The medicinal product which originates from the present invention is intended for the treatment of a cancer in mammals, most especially in humans. The cancers which could be treated in this way are advantageously solid tumours such as breast, lung and colon cancers. It may be noted that such a medicinal product should make it possible, more especially, to treat the secondary tumours which are frequent complications encountered in many types of cancer, and for which no satisfactory therapy exists at present.

The medicinal product which originates from the present invention may be administered according to any general route in common use, in particular parenterally, such as systemically, intramuscularly, subcutaneously or intraperitoneally. Generally speaking, intravenous administration is indicated as being especially advantageous. Alternatively, in the case of an accessible tumour, the medicinal product may also be administered by direct injection into the tumour site or by topical

application. Generally speaking, the administration may take place in a single dose or a dose repeated one or more times after a certain time interval.

According to a preferred embodiment of the invention, the medicinal product will comprise, beside the therapeutically active amount of the said viral vector, a carrier which is acceptable from a pharmaceutical standpoint. It may also comprise a vehicle, a diluent or an adjuvant which is acceptable from a pharmaceutical standpoint, and may be presented in liquid or lyophilized form.

The appropriate dosage varies in accordance with different parameters, for example the administration route, the individual to be treated, the nature and severity of the tumour state, the type of viral vector employed or alternatively the gene or genes coding for the modulatory agent. One of the criteria which enables the appropriate dosage to be evaluated is measurement of the serum activity of the modulatory agent. These tests of activity are standard tests. In particular, there may be mentioned the IL-2 bioactivity test (Gillis et al, 1978, J. Immunol., *120*, 2027-2032). However, in general, the dose of viral vector/kilo will be from 10^4 to 10^{11}, advantageously from 10^7 to 10^{10} and preferably from 10^7 to 10^9 plaque forming units (pfu)/kilo.

Lastly, the subject of the present invention is:

(i) The use of a viral vector displaying a positive tropism for cancer cells, into the genome of which is inserted a DNA fragment containing one or more genes coding for at least one agent participating in the destruction of cancer cells, e.g. an agent that modulates the immune and/or inflammatory response, placed under the control of the elements needed for expression, in particular to deliver the said agent specifically to a cancerous tumour;

(ii) A method for the treatment of cancer in mammals, according to which an individual requiring such a treat-

ment is injected with an amount which is effective, from a pharmaceutical standpoint, of a viral vector into the genome of which is inserted a DNA fragment containing one or more genes coding for at least one agent participating in the destruction of cancer cells, e.g. an agent that modulates the immune and/or inflammatory response; and

(iii) A method for specifically delivering an agent participating in the destruction of cancer cells, e.g. an agent that modulates the immune and/or inflammatory response, according to which a viral vector into the genome of which is inserted a DNA fragment containing one or more genes coding for all or part of the said agent is administered systemically.

The present invention is illustrated, though not limited, by the examples which follow.

EXAMPLES

EXAMPLE 1: Construction of recombinant vaccinia viruses carrying a gene coding for a cytokine

The constructions described below are carried out according to the general techniques of genetic engineering and molecular cloning detailed in Maniatis et al. (1989, Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). All of the cloning steps employing bacterial plasmids are performed using *Escherichia coli* (*E. coli*) strain 5K or XL1-Blue (Stratagene) as host strain, whereas those employing vectors derived from phage M13 are carried out in *E. coli* NM522.

As regards site-directed mutageneses with synthetic oligodeoxynucleotides, the protocol described by Zoller and Smith (1982, Nucleic Acids Res., *10*, 6487) is applied, or a kit of commercial origin is employed according to the manufacturer's recommendations.

The DNA fragments containing the genes coding for GM-CSF, IL-4, IL-5, IL-6 and IL-7 are subjected to a site-directed mutagenesis step so as to introduce the

appropriate restriction sites for the purpose of their insertion into a transfer vector, downstream of the vaccinia virus 7.5K promoter. Two transfer vectors are used: pTG186-poly (described in Patent Application EP 206,920) and pTG194. The latter differs from pTG186-poly in the orientation of the polylinker.

More specifically:

A fragment carrying the cDNA coding for murine GM-CSF, as described in Gough et al. (1984, Nature, *309*, 763), is subjected to a site-directed mutagenesis after cloning into M13TG130 (Kieny et al., 1983, Gene, *26*,91), so as to create a *Bam*HI site at position -17 relative to the translation initiation ATG. The *Bam*HI-*Sal*I fragment isolated from the vector resulting therefrom is inserted between the same sites of pTG194.

An *Eco*RI-*Bam*HI fragment carrying the cDNA coding for murine IL-4, as described in Lee et al. (1986, Proc. Natl. Acad. Sci. USA, *83*, 2061), is introduced into the vector M13TG130 and subjected to a site-directed mutagenesis so as to create a *Bgl*II site immediately upstream of the translation initiation ATG. The vector thus treated is digested with *Eco*RI and *Bgl*II, and the corresponding fragment introduced between the same sites as pTG194.

An *Eco*RI-*Bam*HI fragment carrying the cDNA coding for murine IL-5, as described in Kinashi et al. (1986, Nature, *324*, 70), is first subcloned into the vector M13TG130 and then subjected to a site-directed mutagenesis, so as to introduce a *Pst*I site at position -10 and an A at position -3 relative to the translation initiation ATG. The vector thus treated is digested with *Eco*RI and *Pst*I and the corresponding fragment inserted between the same sites as pTG186-poly.

An *Eco*RI fragment carrying the cDNA coding for murine IL-6, as described in Van Snick et al. (1988, Eur. J. Immunol., *18*, 193), is subcloned into the vector M13TG131 (Kieny et al., *supra*) and subjected to a site-directed mutagenesis, so as to introduce a *Bam*HI site at position -9 and an A at position -3 relative to the

translation initiation ATG. The vector thus treated is digested with *Eco*RI and *Bam*HI, and the corresponding fragment inserted between the *Bam*HI and *Eco*RI sites of pTG194.

An *Sst*I-*Hind*III fragment carrying the cDNA coding for murine IL-7, as described in Naman et al. (1988, Nature, *333*, 571) is subcloned into the vector M13TG130 and subjected to a site-directed mutagenesis, so as to create an *Eco*RV site at position -11 and an A at position -3 relative to the translation initiation ATG. An *Eco*RV-*Pst*I fragment is isolated from the vector thereby obtained, and cloned between the *Sma*I and *Pst*I sites of pTG194.

Using the transfer vectors obtained above, the corresponding vaccinia viruses are produced according to the homologous recombination method described by Kieny et al. (1984, Nature, *312*, 163). The recombinant viruses thereby obtained are designated VV-GM-CSF, VV-IL-4, and the like.

Other recombinant vaccinia viruses have been described previously; in particular, those containing the cDNA coding (i) for human IL-2 (Patent Application EP 206,939), (ii) for human IL-6 (Nakagawa et al., 1991, Eur. Cytokine Net., *2*, 11-16) and (iii) for human IFN-γ (Patent Application EP 206,920).

EXAMPLE 2: Treatment of nude Swiss mice carrying tumours with a vaccinia virus containing the gene coding for IL-6 (VV-IL-6)

1. *Preparation of nude Swiss mice carrying tumours*

The cell line SW948 (ATCC CCL 237) generated from a human colorectal tumour is cultured in continuous fashion according to the supplier's recommendation. The cells which are harvested are treated with trypsin and then with deoxyribonuclease (10 μg/ml) for 5 min. They are then washed in PBS (Dulbecco phosphate buffered saline; Sigma, D5652) and resuspended in this same buffer at a concentration of 10^7 cells/100 μl.

6- to 8-week-old female nude Swiss mice (Iffa Credo, France) each receive subcutaneously 100 µl of the cell suspension thereby obtained. Seven days later, mice carrying palpable tumours are selected.

2. Test

These mice are divided into groups of approximately eight. Two groups are intended to serve as reference groups: the mice receive either 10^7 or 10^8 pfu of a TK- non-recombinant vaccinia virus generated from the transfer vector pTG186-poly (VV-186). The mice of a third group (negative control group) receive only 100 µl of PBS. Lastly, the mice of two other groups receive either 10^7 or 10^8 pfu of the VV-(murine IL-6) obtained in Example 1.

The viruses are administered in PBS solution, intravenously in the tail.

Seven days after injection, 3 mice are removed from each of the groups and sacrificed so as to analyse the viral content of their blood, organs and tumours, as follows.

The tumours and different organs (liver, spleen, brain, and the like), when removed, are treated with trypsin and dislocated mechanically until a suspension is obtained. The blood samples are diluted with an equal volume of PBS buffer comprising 20 mM EDTA.

The cells thereby obtained are washed twice in PBS and resuspended in Dubelcco [sic] MEM culture medium (Modified Eagle's Medium; Gibco BRL) comprising 10% of foetal calf serum. The number of cells (viable and non-viable) is estimated by counting according to conventional methods. 10-fold serial dilutions are then performed in PBS buffer. 1 µl, 10 µl or 100 µl of each of the dilutions is/are added to cultures of BHK cells established in Petri dishes 3 cm in diameter (Falcon 3001). The dishes are incubated overnight at 37°C in 5% CO_2, and the lytic plaques counted on the following day.

As regards the mice not sacrificed, the course of the growth of the tumours is recorded over time by

measuring the length, width and depth of the tumours using a sliding caliper. The volume of each tumour is calculated by applying the formula for ellipsoids $4/3\pi r_1 r_2 r_3$, in which r_1, r_2 and r_3 represent the length, width and depth, respectively reduced by one half.

3. *Results*

Irrespective of the type of virus injected, it is found that the level of infection of the tumour cells is much greater than that of the healthy tissues.

The growth of the tumours of the mice which received VV-IL-6, irrespective of the dose, is less than those of the control groups and the negative reference. Some mice display a complete regression of the tumours approximately 15 days after the administration of VV-IL-6.

EXAMPLE 3: Treatment of DBA/2 mice carrying tumours with a vaccinia virus containing the gene coding for IL-2 (VV-IL-2)

Example 2 is repeated using:

(i) the vaccinia virus containing the gene coding for human IL-2, as described in Patent Application EP 206,939;

(ii) the murine cell line P815 (ATCC TIB 64) originating from a DBA/2 mouse mastocytoma; and

(iii) 6- to 8-week-old female DBA/2 mice (Iffa Credo, France).

The variants are as follows: 10^5 P815 cells in a volume of 100 µl are injected into the DBA/2 mice. 10^6 pfu of virus are injected into the mice of each of the groups. The mice intended for biological analyses are removed from the groups 3 days after injection.

Results comparable to those reported in Example 2 are observed.

EXAMPLE 4: Treatment of DBA/2 mice carrying tumours

with a vaccinia virus containing the gene coding for GM-CSF (VV-GM-CSF)

Example 3 is repeated using 10^8 pfu of VV-GM-CSF. The course of the growth of the tumour is recorded over time as described in Example 1. As before, a slowing of the growth of the tumours relative to the control groups and the negative reference is observed in some animals of the test group. Three out of 10 mice display a complete regression of the tumours 15 days after administration of the viral vector.

The claims defining the invention are as follows:

1. Use of a viral vector in the genome of which is inserted a DNA fragment containing one or more genes coding for all or part of an agent that modulates the immune and/or inflammatory response, wherein said agent is:

of cell origin;

selected from cell surface costimulatory molecules, chemokines, monoclonal antibodies directed against lymphocytes surface markers, superantigens characteristic of an infectious organism (bacterium, virus or parasite) and polypeptides having an adjuvant function, for the preparation of a medicinal product for parenteral administration, and in particular intravenous or intramuscular administration, for the treatment of a declared cancer in mammals.

2. Use of a viral vector according to Claim 1 according to which the cell surface costimulatory molecules are selected from classes I and II antigens of the major histocompatibility complex (MHC), ligands for the markers CD27, CD28, CD30 and CD40, the lymphocyte function antigen type 1 (LFA-1) and the intercellular adhesion molecule type 1 (ICAM-1).

3. Use of a viral vector according to Claim 1 according to which the chemokines are selected from PF-4 (platelet factor-4), RANTES and ACT-2.

4. Use of a viral vector according to Claim 1 according to which the monoclonal antibodies are selected from anti-CD2, anti-CD3, anti-CD28 and anti-CD40 monoclonal antibodies.

5. Use of a viral vector into the genome of which is inserted a DNA fragment containing one or more genes coding for at least one cytokine, for the preparation of a medicinal product for the treatment of a declared cancer in mammals; the said medicinal product being intended for parenteral

administration, and in particular for intravenous or intramuscular administration.

6. Use of a viral vector according to Claim 5 according to which the cytokines are selected from interleukins, interferons, colony stimulating factors (CSF), tumour necrosis factors (TNF), macrophage migration inhibition factors (MIF) and complement fragment C5a.

7. Use of a viral vector according to Claim 6 according to which the cytokines are selected from interleukins-2, -4, -5, -6 and -7, gamma interferon, granulocyte-macrophage type colony stimulating factor (GM-CSF) and tumour necrosis factor type β (TNFβ).

8. Use of a viral vector according to one of Claims 1 to 7, for the preparation of a medicinal product for the treatment of a declared cancer in humans.

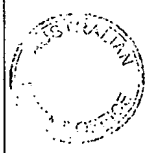
9. Use of a viral vector according to one of Claims 1 to 8, for the preparation of a medicinal product for the treatment of a solid cancerous tumour in mammals.

10. Use of a viral vector according to one of Claims 1 to 9, according to which the viral vector is derived from a virus selected from poxviruses, adenoviruses, retroviruses and herpesviruses.

11. Use of a viral vector according to one of Claims 1 to 10, according to which the viral vector is a non-integrative vector.

12. Use of a viral vector according to one of Claims 1 to 11, according to which the viral vector is non-replicative vector.

13. Use of a viral vector according to Claim 10, according to which the viral vector is derived from a poxvirus.



14. Use of a viral vector according to Claim 13, according to which the viral vector is derived from an avian poxvirus.

15. Use of a viral vector according to Claim 13, according to which the viral vector is derived from a vaccinia virus.

16. Use of a viral vector according to one of Claims 1 to 15, according to which the viral vector comprises, in addition, a gene coding for a tumour-specific antigen.

17. Use of a viral vector displaying a positive tropism for cancer cells, into the genome of which is inserted a DNA fragment containing one or more genes coding for at least one agent participating in the destruction of cancer cells, wherein said agent is of cell origin and placed under the control of the elements needed for expression, to deliver the said agent specifically to a cancerous tumour.

18. Use according to claim 17 wherein said agent is one or a plurality of cytokines.

19. Use according to claim 18 wherein said cytokines are selected from interleukins, interferons, colony stimulating factors (CSF), tumour necrosis factors (TNF), macrophage migration inhibition factors (MIF) and complete fragment C5a.

20. Use according to claim 18 wherein said cytokines are selected from interleukins-2, -4, -5, -6 and -7, gamma interferon, granulocyte-macrophage type colony stimulating factor (GM-CSF) and tumour necrosis factor type β (TNFβ).

21. Use of a viral vector according to any one of claims 17 to 20, into the genome of which is inserted a DNA fragment containing one or more genes coding for an agent that modulates the immune and/or inflammatory response, placed under the control of the elements needed for expression, to deliver the said agent that modulates the immune and/or inflammatory response specifically to a cancerous tumour.

22. Use of a viral vector according to Claim 17 or 21, according to which the viral vector is derived from a poxvirus.

23. Use of a viral vector according to any one of claims 1 to 22 substantially as hereinbefore described with particular reference to the examples.

DATED: 24 June 1999

CARTER SMITH & BEADLE
Patent Attorneys for the Applicants:

TRANSGENE S.A.